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THE INTRACUTANEOUS 'TYPHOIDIN' REACTION *

I. THE PREPARATION AND PROPERTIES OF THE ANTIGEN

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Thus far no decision has been reached regarding the value of the 'typhoidin' skin reaction as a means of determining the immunity which develops after typhoid infection or prophylactic inoculation with vaccines.

Gay and his co-workers[1] still adhere to their original conception that the typhoidin or paratyphoidin skin reactions are specific and indicative of an existing immunity against typhoid and allied fevers. Pulay,[2] and also Kilgore[3] in his first paper, have been the only recent supporters of this interpretation of the typhoidin skin reaction. Evidence, however, has gradually been collected by Nichols,[4] Austrian and Bloomfield,[5] Kolmer and Berge,[6] Kilgore,[7] and others, which would support the view that the skin reaction is either a true anaphylactic phenomenon—the expression of a cutaneous sensitization to typhoid protein—or a nonspecific dermatic response of the human skin to toxic elements contained in the typhoidin liquid or powder. Kolmer and Berge[6] consider the reaction an indicator of typhoprotein sensitization, but state that "there is not yet sufficient evidence to warrant its acceptance as an index of immunity in typhoid fever." In similar studies made with 'luetin' and 'diphtherin' skin reactions, they were compelled to the conclusion that these skin tests cannot be used to determine in man the existence or absence of a defensive activity against the respective diseases.

Following the discovery of von Pirquet's cutaneous tuberculin test in 1907, analogous investigations were carried out as to the diagnostic value of the typhoidin reaction in typhoid fever. Chantemesse,[8] Zupnik,[9] Link,[10] Deehan,[11] Floyd and Barker,[12] and Austrian,[13] reported favorably on the value of the cutaneous and ophthalmic reactions with various kinds of typhoid preparations.

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* Received for publication December 4, 1916.

[1] Arch. Int. Med., 1914, 13, p. 471; 14, p. 697.

[2] Wien. klin. Wchnschr., 1915, 28, p. 1382.

[3] Arch. Int. Med., 1916, 17, p. 25.

[4] Jour. Exper. Med., 1915, 22, p. 780.

[5] Arch. Int. Med., 1916, 17, p. 663.

[6] Jour. Immunol., 1916, 1, p. 409.

[7] Arch. Int. Med., 1917 (still to be published).

[8] Deutsch. med. Wchnschr., 1907, 33, p. 1572.

[9] München. med. Wchnschr., 1908, 55, p. 148.

[10] Ibid., p. 730.

[11] Univ. of Penn. Med. Bull., 1909-1910, 22, p. 192.

[12] Am. Jour. Med. Sc., 1909, 138, p. 188.

[13] Bull. Johns Hopkins Hosp., 1912, 23, p. 1.

Kraus,[14] Wolff-Eisner,[15] Goodmann and Sutter,[16] Chauffard and Troisier[17] on the other hand, failed to confirm their statements, and questioned the specificity of the reactions. Kraus, Lusenberger, and Russ,[18] Entz,[19] Rolly,[20] and von Szontagh[21] showed that a high percentage of normal individuals also react to various bacterial toxins, and among them the typhoid toxin. It would appear that very little attention has been paid to all these studies, tho it is quite apparent that the typhoidin powder now used contains most of the substances which, in the observations of Entz, Kraus, and others, must have given rise to nonspecific skin reactions in man. That not all the skin reactions are true anaphylactic or, as in the Schick test, true antitoxin-toxin neutralization phenomena, is pointed out by Besseau and Schwenke[22] in a recent report on the local reaction in children to diphtheria broth. They consider the coctostabil endotoxin of the diphtheria bacillus the substance which caused the skin efflorescence in a certain percentage of children injected intracutaneously with neutralized toxin-antitoxin mixtures. In their opinion the irregular susceptibility of man to these substances and the absence of this type of sensitiveness in animals make further investigations desirable, inasmuch as cutaneous tests are now more and more used for diagnostic and experimental purposes.

Our studies on the nature, mechanism, and value of the typhoidin reaction are part of extensive observations on typhoid infection in rabbits.

An opportunity to solve some of the problems presented was afforded in the availability of animals which had been used in the preparation of various antisera and in other immunologic experiments.

An adequate presentation of this subject would require a discussion of the basic principles underlying skin reactions in general and their relation to anaphylaxis, infection, and immunity. In the first of these communications we wish to confine our attention to the methods of preparation and standardization of typhoidin and similar glycerin extracts, leaving a consideration of the other problems to the papers which follow.

METHODS OF PREPARATION OF TYPHOIDIN AND SIMILAR GLYCERIN BROTH-CULTURE EXTRACTS

Little attention has been paid to the exact mode of preparing typhoidin. The literature on this subject states only incompletely the composition and the nature of the peptones used in the medium. In fact, some workers have failed to emphasize one of the most important points underlying its preparation; namely, that the typhoidin thus far used is a glycerin extract of the typhoid bacillus, and that, as is well known from extensive studies on tuberculin, mallein, 'abortin,' and the like, such extracts contain a large amount of salts and other extractives. It is also a well-established fact that broth cultures, or autolysates and similar preparations of pure bacterial protein, not extracted with glycerin, give less distinct and consistent reactions than do glycerin extracts; and yet, for local anaphylactic reactions tuberculin or mallein is more efficacious if it contains elements of the bacterial bodies (Klimmer[23]).

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[14] Wien. klin. Wchnschr., 1907, 20, p. 344.

[15] Die Ophthalmo- und Cuti-diagnose der Tuberculose. 1908.

[16] Univ. of Penn. Med. Bull., 1909-1910, 22, p. 81.

[17] Compt. rend Soc. de biol., 1909, 66, p. 519.

[18] Wien. klin. Wchnschr., 1907, 20, p. 1385. München. med. Wchnschr., 1910, 57, p. 437.

[19] Ibid., 1908, 21, p. 379.

[20] München. med. Wchnschr., 1911, 58, p. 1285.

[21] Arch. f. Kinderh., 1912, 58, p. 326.

[22] Monatschr. f. Kinderh., 1915, 13, p. 397.

For cutaneous or ophthalmic reactions, workers have found it preferable to remove the glycerin and to purify the various tuberculin and mallein extracts by precipitation with alcohol. Since the latter procedure is sometimes attended with difficulties, various other means have been devised to obtain a suitable powder. The procedure followed by us will be discussed in detail in subsequent paragraphs.

It has been customary to consider the typhoidin a preparation analogous to tuberculin, but we feel that such a conception is not fully correct, because the metabolism of the tubercle bacillus and that of the typhoid bacillus differ so widely that it is not unreasonable to assume that their glycerin-broth cultures must contain catabolic and anabolic products differing greatly in character. That this is the case is shown by the experience that concentrated tuberculin remains active for more than one year, but that a similar typhoidin specimen is usually inert after one month. Furthermore, we know nothing about the value of the toxic pyrogenic substances and their gradual distintegration in typhoidin. In the case of mallein, however, one of us[24] has found that an old powder, practically free from nonspecific toxic elements, gives very selective and, from a diagnostic viewpoint, better results than do fresh, highly toxic solutions. In order, therefore, to determine the value of typhoidin as an aid in immunologic research, it is of the greatest importance that these points be carefully investigated.

In the present series of experiments we have employed glycerin extracts of broth cultures, reserving for future investigation the value of pure bacterioprotein as an antigen.

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[23] Handbuch der Serumtherapie und Serumdiagnostik in der Veterinär Medizin, 1911, p. 109.

[24] Meyer, Jour. Infect. Dis., 1913, 12, p. 171.

In previous reports on typhoidin we failed to find a reference to the standardization of the product, especially in so far as its antigenic properties are concerned. Furthermore, no explanations are given in the literature to account for the well-known observation that old preparations are inactive. We therefore felt that these and other questions required further study.

General Technic for the Preparation of Typhoidin.—Erlenmeyer flasks containing 500 c.c. of a veal infusion (1% peptone; 0.5% sodium chlorid; 5% glycerin) of a reaction varying from 0.5 to 1 (pH^+ 7.4 to 7.6) were inoculated with one strain of B. typhosus. The flasks, loosely capped with tinfoil, were incubated for varying periods at 37 C., and then tested for purity. One-half of the culture was, without previous sterilization, concentrated to one-tenth of its volume on the water-bath at a temperature which rose gradually from 40 C. to 60 C.

The syrupy milky fluid, after it had stood from 24 to 48 hours, was centrifugated at low speed to remove some of the phosphates. Usually no attempt was made to clear it. All the manipulations of concentrating, etc., were done in diffused light or in the dark. The final product was kept in brown bottles in the refrigerator until ready for precipitation.

From 10 to 20 c.c. of the concentrated typhoidin were precipitated in from 200 to 400 c.c. of absolute alcohol. It is important that the alcohol be absolutely water-free and that the syrupy typhoidin be added to the alcohol only by drops. In case the milky mixture does not flocculate in a few minutes, a few sodium chlorid crystals should be added. As soon as the coarse light-yellowish precipitate has settled in the ice-chest, the supernatant alcohol is siphoned off and replaced by fresh absolute alcohol. Then the suspension is quickly centrifugated, the alcohol poured off, and the sediment suspended in a very small amount of ether-alcohol. Finally, the voluminous precipitate is filtered off, collected on fresh sterile filter paper or clay plates, and dried in vaccuo over sulfuric acid or fresh calcium chlorid. Several preparations were redissolved in a 1% sodium-chlorid solution and precipitated again. Perfectly white or light-brownish granular powders were finally obtained. Such preparations are slightly, if at all, hygroscopic, but are soluble in salt solution; for example, a portion of the preparation Typhoidin B15 was kept for 4 weeks in a room in which steam from sterilizers added considerably to the high humidity of the foggy climate. The powder did not cake, and remained granular. Moisture was absorbed according to the increased weight of the powder and, together with the effect of the diffused light, was probably responsible for the deterioration and complete loss of antigenic properties of this test sample of typhoidin.

As a rule, these powders dissolved readily in salt solution forming turbid or opalescent solutions or suspensions in which the bacterial débris usually settled in the form of a grayish sediment. The repeated treatment with alcohol made these powders insoluble, however, so that only suspensions could be obtained. It is not unlikely that such preparations, on account of their colloidal nature, act as irritants, and are probably responsible for the nonspecific reactions. To overcome this possible insolubility of the typhoidin powders, we would recommend the filtration of the concentrated extracts, and the careful use of absolute alcohol. One should particularly avoid repeated washings and a too long contact of the preparation with alcohol.

All typhoidin powders were kept in brown bottles, protected from the light in desiccators at room temperature. Only in special experiments were polyvalent glycerin extracts used; in the majority of instances the typhoidin preparations were monovalent. Shortly before use, definite amounts were dissolved in carbolized (0.5%) salt solution. The glycerin broth-culture extracts of various experiments were prepared in the same manner. The broth used for the control powders was incubated with the cultures and handled absolutely in the same manner as that outlined. It was in this connection that we first found that repeated treatment with alcohol produces insoluble powders.

We found also that the composition of the culture medium and its reaction have a considerable influence on the preparations obtained. It will be shown (Experiment-group 2) that a 2% peptone medium or Martin's broth gives a very toxic typhoidin, and that a potato-glycerin-glucose-Lemco-broth* is better than veal broth. The type of peptone also influences the toxicity of the typhoidin; peptone-Chapoteaut gives a somewhat more toxic powder than Witte's peptone. An optimal reaction of the culture fluid was always chosen and was found to be about pH^+ 7.4 to 7.6, as determined by our colorimetric method.[25]

It will be shown later that the amount of broth inoculated with the typhoid strains is also of importance in the preparation of a good product. If the broth is kept in a layer of 2 cm., the toxicity is remarkably increased. In small amounts the sterile filtrate is toxic to rabbits on intravenous inoculation, and the number of skin reactions on normal rabbits is strikingly increased. To overcome this difficulty, we grew the bacteria in flasks as completely filled as possible. We have also chosen the glycerin potato broth and prolonged incubation (20 to 30 days) to overcome some of the factors which we believe impart to the typhoidin preparations a high degree of primary toxicity. A more extensive discussion of these important details will be undertaken in connection with the experimental data presented in later paragraphs.

Standardization of Typhoidin and Similar Preparations. — As a result of our experience with abortin, reported in 1913,[26] in which we followed the suggestions of Calmette and Massol[27] on tuberculin, we have chosen the complement-fixation test as a means of determining the exact amount of specific antigenic substances in a given preparation. Preliminary tests had already indicated that neither the general appearance of the typhoidin nor its method of preparation have much influence on its antigenic properties. On the other hand, it is generally conceived, from some work done with mallein, that the diagnostic value of a preparation depends largely on its antigenic units. This fact will be more fully considered and illustrated by subsequent experiments.

In general the antigenic value of a preparation may be ascertained either by complement-fixation or by inoculation tests.

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* Raw potato (500 gm.) in 2000 c.c. of water, is macerated for 12 hours at 60 C., strained, and 10 gm. of meat extract (Lemco), 10 gm. of salt, and 20 gm. of peptone are added, the whole steamed, rendered faintly alkaline to litmus (pH^+ 7.4) filtered after steaming for 1½ hours, and 1% glucose and 1% glycerin added before distribution in flask.

[25] Bull. Johns Hopkins Hosp., 1916, 27, p. 16.

[26] Jour. Infect. Dis., 1913, 13, p. 355.

[27] Compt. rend. Soc. de biol., 1912, 72, p. 15.

TABLE 1

STANDARDIZATION OF TYPHOIDIN BY MEANS OF COMPLEMENT-FIXATION TESTS

Tube	Typhoidin 17, Diluted 1:100	Rabbit Immune Serum 2×Titer	Complement 1:4	Saline Solution (0.85%)	Antisheep Hemolysin (1:100)	Blood-cell Suspension (1%)	Result
1	0.01	0.001	0.05	1.5	0.2 (twice the hemolytic unit)	0.5	No hemolysis
2	0.0075						
3	0.005						
4	0.0025						
5	0.002						
6	0.001						50% hemolysis
7	0.00075						Hemolysis
8	0.0005						
9		0.1					
10	0.01	0.001					
11	0.0075						
12	0.005	normal serum					
13	0.0025						
14		0.1 normal serum					
15	0.1						
16	0.05						
17	0.01						
18	0.0075						
19	0.005						
20							

The technic of complement-fixation for this particular purpose deviated very little from the method employed in our laboratory and recently described in detail.[28] Double amounts of a highly potent rabbit typhoid-immune serum, prepared with the same strains which had been used for the manufacture of the typhoidin, were titrated against descending dilutions of the powder. The powder was usually dissolved in 100-mg. doses in 10 c.c. of carbolized salt solution. An antisheep hemolytic system was used as indicator. In Table 1 a typical determination is given.

Table 1 shows that the smallest amount of typhoidin causing fixation of complement, is 0.002 c.c. of a solution of 0.1 gm. of typhoidin in 10 c.c. of salt solution. Neither nonspecific fixation nor hemolysis was observed in the control tubes of the serum or the antigen with and without normal serum. The amount of 0.002 c.c. was designated by us as an extract-unit (e.u.), and we could easily determine the number of such units per 10 c.c. or for 0.1 gm. of a given typhoidin. For purposes of clarity, the method of deriving the result of the example cited in Table 1 may be given. Thus the proportion would be: 10:0.002, which gives 5000 units per 0.1 gm. or 50 units per 0.001 gm.—the dosage usually employed in our experiments. A preparation containing about 20 to 40 e.u. may be considered a good product. The strength of the preparation gradually diminishes in salt solution, but with the method just outlined it was possible to determine, with little difficulty, the relative quality of the typhoidin preparation chosen for some of the experiments.

In Table 2 some of the unit determinations are classified to illustrate the value of the method as a guide in anaphylactic skin reactions. The results recorded for Nos. 3 and 4 explain the observation that preparations older than 4 to 6 months are poor reagents in animals. That some of the reactions obtained in man with such old typhoidin preparations are doubtless not due to the antigenic substances of the preparations, but to some nonspecific irritant, is made also more plausible by these tests.

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[28] Jour. Exper. Med., 1916, 24, p. 516. Arch. Int. Med., 1917 (still to be published).

TABLE 2

CLASSIFICATION OF UNIT DETERMINATIONS FOR TYPHOIDIN

No.	Preparation	Appearance	Smallest Dose which Completely Fixes the Anti-serum	Extract Units per 0.001 gm.
1	Typhoidin 3. Veal infusion + 1% Chapoteaut-peptone. Strain Olsen, fresh	Coarse light-brownish powder, readily soluble	0.001	100
2	Typhoidin 3, dissolved in carbolized salt solution 1:100 and kept at ice-chest temperature for 22 days	Light-brownish fluid with slight grayish sediment	0.005	20
3	Typhoidin 2. Veal infusion + 1% Chapoteaut-peptone. Strain Dorset. Reprecipitated Dec. 15, 1915	Light whitish powder, readily soluble	0.003	33.3
4	Typhoidin 2; freshly dissolved powder June 7, 1916	Light-yellow turbid fluid	0.075	1.3
5	Typhoidin 4. Veal infusion + 1% Witte's peptone. Strain Kuhn. Incubation 48 hours, fresh	Slightly caked, but readily soluble, powder; light-brown	0.01	10
6	Typhoidin 4, 2 months later	Same powder	0.025	4
7	Typhoidin 17 (Olsen). Potato glycerin broth fresh on March 28, 1916	Finely granular deep-brownish powder, soluble with a considerable grayish sediment	0.002	50
8	Typhoidin 17. June 7, 1916	Fresh solution	0.003	33.3
9	Martin's-broth typhoidin (Olsen)	Fine powder, easily soluble to an opalescent fluid	0.005	20
10	Distilled residue of wash alcohol of Typhoidin B	Smeary gummy substance, soluble in salt solution	0.1	1

It is well to bear another important point in mind, namely, that repeated resolution and reprecipitation of the typhoidin produces a preparation poor in extract units, the residue of the wash alcohol after distillation giving, when dissolved in salt solution, fixation of the complement.

From our tests it is not possible to state with certainty that the latter is due to protein substances or to the lipoid-like alcohol-soluble material of the typhoidin. Whenever possible, the antigenic properties of other bacterial extracts were tested by complement-fixation, but our main experience for the most part is based on the work with typhoidin.

Finally, the antigenic value of some of the typhoidin preparations was also tested by direct intravenous injections of rabbits with 0.1 to 0.01 gm. of powder dissolved in salt solution. The results with 3 typhoidin preparations are shown in Chart 1.

As a rule, recently isolated strains of B. typhosus give a typhoidin poor in antigenic properties (Curve 3). Repeated purification reduces somewhat the agglutinin-stimulating effect of the typhoidin powder (Curve 2).

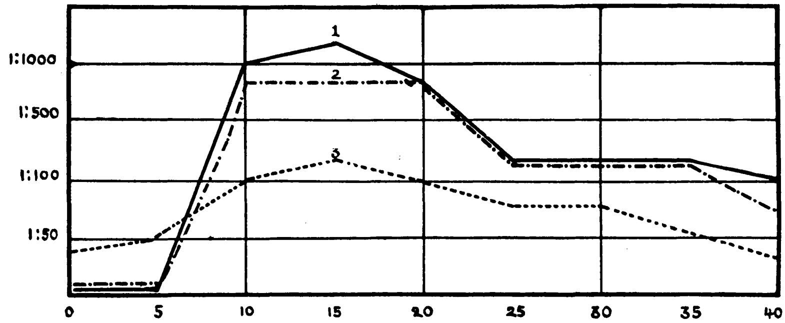

Chart 1. The average agglutination titers of 6 rabbits inoculated with 0.01 gm. of typhoidin.

———————— 5-day-old culture of an old strain, not filtered or purified.

—.—.—.— 5-day-old culture of the same strain, purified by 3 successive precipitations.

--------- 5-day-old culture of a recently isolated strain, not purified.

MODE OF APPLICATION OF THE GLYCERIN EXTRACTS

All tests were carried out by the intracutaneous inoculation of 0.001 to 0.0001 gm. of the powder dissolved in carbolized salt solution. The dilutions of the typhoidin preparations were so chosen that 0.1 c.c. contained the amounts of antigenic substance stated. Only fresh solutions or suspensions of the powder were used. The skin of the back was shaved from 2 to 3 days previous to the tests, and was carefully freed from the irritant soap and cleansed with alcohol before the injections were made. Platinum-iridium needles and Record syringes were used throughout the tests. Some experience, to be sure, is required in applying the suspensions intracutaneously, but we cannot agree with Kolmer[29] that the intracutaneous applications of test fluids is any more difficult in the rabbit than in the guinea-pig or any other laboratory animal.

Ordinarily 2, sometimes from 3 to 4, different extracts in various dilutions were tested on the same animal. Each preparation was compared with its control powder made from the sterile culture medium. Naturally, sites of injection were always chosen as far apart as possible so as to avoid fusion of the reactions. In this we were successful in the majority of experiments, but in some the toxicity of the preparations resulted in such a marked edema that complete separation of the reaction areas was not possible.

REACTIONS CAUSED BY THE EXTRACTS

It is perhaps not generally known that rabbits are not very suitable for skin tests. It may be well to recall the fact that tuberculous animals of this species react slightly, if at all, to the ordinary tuberculin test, but fairly well to the intracutaneous or subcutaneous application of a suspension of dead tubercle bacilli (Klimmer[30]). Experience with this reaction has taught us that a positive result is characterized by a red areola surrounding the needle puncture, with marked swelling, induration, and increased heat at the reacting area — these features becoming more striking usually within 24 hours, and frequently lasting from 2 to 3 days.

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[29] Jour. Amer. Med. Assn., 1916, 67, p. 719.

[30] Handbuch d. Serumtherapie, etc., 1911, p. 120.

Similarly, a specific reaction with typhoidin is, in our experience, characterized by an area of erythema and a central indurated papule. The diameter of the areola may vary from 1 to 3 cm., depending on the concentration of the antigenic extract and the time interval after the injection. Under ordinary conditions the maximum of this reaction is reached in about 24 to 36 hours after the injection. If at the end of this period the longest diameter of the areola measures 10 mm. or more, the reaction may be regarded as positive. A positive reaction persists to the 40th hour and begins to disappear very gradually, so that at about the 72nd hour only faint outlines of the areola and papule can be noticed. As a rule, the reading at the end of 24 hours shows a greater intensity of reaction than after 48 hours. Sometimes delayed reactions reach their height only at or after 48 hours. This makes readings after 72 hours essential. A response—probably not due to an antigen-antibody reaction—appears more rapidly in the first 24 hours and fades with equal rapidity. The diameter of the areola may be the same as in a positive reaction, but the edema and hyperemia are less marked, and particularly the papule-formation is, for the most part, absent. Such reactions have been recorded in the experimental data as "red areas." The control extracts usually cause a small areola of a maximal diameter of 0.5 cm., and in these instances only a reddish spot with very little induration is noticed. These are referred to as "red spots," or, whenever smaller, as "needle puncture." In a few instances very marked edema and infiltration of the skin with little or no hyperemia were observed in control animals. Such reactions are difficult to explain, but in our experience they are found only when the preparations are rich in colloidal substances, or they may be the result of faulty technic. The yellowish center in the papule, or nodule, develops only when highly concentrated extract solutions are used, or if the needle puncture has become infected with skin organisms, or if the test powder has become contaminated.

The selection of typhoidin dilutions gave us an opportunity to work quantitatively and, in numerous instances, this was of extreme value in forming an opinion as to the specificity of a reaction. When a preparation was found to be very irritant to the rabbit in high concentrations, as was apparent from the reaction it produced in normal animals, a dilution was then chosen which gave a negative result. Such dilutions gave, as a rule, well-marked reactions in sensitized or immune animals, and the intensity of the areola persisted usually for at least 48 hours. Furthermore, the difference in the diameters of the indurated areolae of the various dilutions in comparison with the control spot were usually so striking that they assisted greatly in forming judgments concerning positive or negative reactions. For similar reasons we tested usually from 3 to 4 different preparations and their corresponding controls, on the same animal.

For the typhoidin test it was not difficult, in most instances, to interpret the reactions obtained in the immunized animals; the difference between the reactions caused by the typhoidin, or other extracts, and those produced by the control powders, was always sufficiently well marked. This observation is in striking contrast to the well-established fact that the human skin very frequently reacts just as intensively to the control powder as to the typhoidin and, therefore, presents extreme difficulties in the interpretation of the reactions (Kolmer[6]).

To obtain comparative data the diameters of the indurated areolae were measured with a machinist's vernier gauge at intervals of 16, 24, 48, and 72 hours after the intracutaneous injections. In the records of the experiments the figures tabulated represent the carefully measured diameters of the areolae expressed in centimeters.

ANIMAL EXPERIMENTS

The various intracutaneous tests with bacterial glycerin extracts were made on about 100 immunized, and 30 normal rabbits.

Immunization.—The method of immunization varied according to the antigens used. A discussion of this point will therefore be deferred until the separate experiments are considered. At least one or more months elapsed before the animals were tested. In a few cases an injection of living organisms was given between the 1st and 2nd test. This was done to test the resistance of these animals, as well as with a view to producing carriers. Some animals were extensively immunized with living and dead bacteria; in a few cases as many as 17 injections were given.

The degree of immunity was observed in the appearance and persistence of agglutinins and complement-fixing antibodies, a large number of animals being tested regularly at 10-day intervals. The sera were collected a day previous to the application of the allergic skin tests and the necessary tests were performed within the next 48 hours.

Agglutination Tests.—The macroscopic technic with standardized, formalinized, or carbolized broth cultures was used. Each rabbit serum was tested with the strain of B. typhosus with which it had been immunized. The control animals and those treated with bacteria other than typhoid were tested either with their respective organisms, or with the typhoid strain used in the typhoidin preparation.

Complement-Fixation Tests.—Complement-fixation tests were conducted with an antisheep hemolytic system and the corresponding broth-culture antigens. To these, references have been made in previous communications.[28] Heated and carbolized 18-hour-old Liebig's-broth cultures were used, for the most part, in case of the typhoid bacillus and allied organisms. Suspensions or aqueous extracts were employed in a few instances only. The antigens were used in amounts corresponding to one-fourth to one-third of their anticomplementary units.

Preliminary experiments on rabbits had demonstrated that the concentration of the typhoidin powder suspended or dissolved in carbolized salt solution influenced, as might be expected, the degree of reaction. A series of experiments was planned, therefore, to determine the amount of typhoidin necessary to produce characteristic reactions in highly immune animals. Furthermore, to test the specificity of the reactions, an extract of the fowl-typhoid bacillus was used as a control. Theobald Smith and TenBroeck have recently shown the close relationship of the bacillus of fowl typhoid and B. pullorum to the human-typhoid bacillus, and our studies along similar lines have confirmed their statements, namely, that the fowl-typhoid bacillus in broth media produces a strong toxin for rabbits. It was therefore of interest to determine whether the rabbit skin is sensitive to these nonspecific substances and whether the reactions obtained with them are distinguishable from the specific skin reactions obtained with typhoidin.

Some of these results are discussed in experiments of Groups 1 and 2.

EXPERIMENT-GROUP 1

Twenty-one rabbits were immunized by various methods. The following organisms were used: B. typhosus; the fowl-typhoid bacillus; B. pullorum; and B. dysenteriae. Four normal animals served as controls. These were tested on Jan. 31, 1916, with a glycerin-extract typhoidin and an identical preparation produced with the fowl-typhoid bacillus.

Nine rabbits (Nos. 314, 317, 319, 321, 323, 324, 325, 326, 333) were intensely immunized against various recently isolated strains of B. typhosus (for most of which we are indebted to Dr. F. P. Gay); 7 injections, at weekly intervals, were given intravenously until Nov. 8, 1915. Judged by serum reactions, most of the animals still showed, 84 days after the last injection, a high degree of immunity. Three rabbits (317, 319, 321) were reinoculated 9 days previous to the test, with one-half blood-agar slant, of the same typhoid strain with which they had been immunized.

Four rabbits (339, 340, 341, 576) were immunized with 3 doses of Army vaccine (obtained through the courtesy of Major H. J. Nichols, of the U. S. Army

TABLE 3

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN
DATE OF TEST: MAY 19, 1916

Animal	Immunization	Last Injection (1915)	Serologic Tests Jan. 27, 1916		Hours	Typhoidin 3	
			Agglutination	Complement-Fixation		1:100	1:500
						(cm.)	(cm.)
314	B. typhosus Rawlings, 7 injections	Nov. 8	1:2000	>0.003	18	2 x 2, W. D.	1.6 x 1.6, W. D.
					28	1.6 x 1.6	1.2 x 1.2
					54	0.5 x 0.5, nodule	N. P.
323	B. typhosus 16, 7 injections	Nov. 8	1:800	>0.01	18	1.9 x 1.9, D. O.	0.2 x 0.2
					28	1.8 x 1.8	Sl. red spot
					54	0.4 x 0.4	N. P.
324	B. typhosus 17, 7 injections	Nov. 8	1:2000	0.003	18	1.9 x 1.9, W. D.	N. P.
					28	2.1 x 2.1, W. D.	N. P.
					54	0.5 x 0.5	N. P.
325	B. typhosus 18, 7 injections	Nov. 8	1:800	>0.01	18	2.2 x 2.2, W. D.	1.0 x 1.9, W. D.
					28	2.2 x 2.2, W. D.	1.7 x 1.7, W. D.
					54	1.3 x 1.3, W. D.	0.5 x 0.5, nodule
326	B. typhosus 19, 7 injections	Nov. 8	1:1000	>0.01	18	1.8 x 1.8, W. D.	1.3 x 1.3, R. I.
					28	1.3 x 1.3, W. D.	0.8 x 0.8, W. D.
					54	0.1 x 0.1	N. P.
333	B. typhosus 47, 7 injections	Nov. 8	1:1000	0.005	18	2 x 2, R. I.	0.8 x 0.8, R. I.
					28	1.8 x 1.8, R. I.	0.2 x 0.2
					54	N. P.	N. P.
339	Vaccine A (usual strength)	Nov. 30, 48 days after	1:2000	<0.003	18	1.8 x 1.8, W. D.	1.3 x 1.3, D. O.
					28	1.8 x 1.8, W. D.	0.7 x 0.7
					54	1.5 x 1.5, W. D.	N. P.
340	Vaccine A	Nov. 30, 48 days after	1:4000	0.005	18	2.4 x 2.4	1.4 x 1.4
					28	2.2 x 2.2	1.1 x 1.1
					54	N. P.	N. P.
341	Vaccine A	Nov. 30	1:4000	0.005	18	2.2 x 2.2, W. D.	0.9 x 0.9
					28	1.9 x 1.9, W. D.	0.5 x 0.5, R. I.
					54	1.3 x 1.3, S. I.	N. P.

KEY TO TABLES

W. D. = well-defined areola
R. I. = deep purplish, indurated area
S. I. = very slight induration
D. O. = diffuse edema
S. O. = slight edema

TABLE 3.—(*Continued*)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN
DATE OF TEST: MAY 19, 1916

Typhoidin 3	Fowl-Typhoidin 3		Control Powder 3	Remarks
1:1000	1:100	1:1000		
(cm.)	(cm.)	(cm.)	(cm.)	
0.3 x 0.3, R. I.	1.9 x 1.9	1.4 x 1.4, R. I.	0.1 x 0.1, S. O.	Reaction very distinct Re-injected April 25, 1916 Not a carrier
N. P.	1.8 x 1.8	0.5 x 0.5	N. P.	
N. P.	0.2 x 0.2, nodule	N. P.	N. P.	
N. P.	D. O.	N. P.	N. P.	Re-injected April 25, 1916 Not a carrier
N. P.	0.7 x 0.7	N. P.	N. P.	
N. P.	0.1 x 0.1, R. I.	N. P.	N. P.	
N. P.	0.6 x 0.6	N. P.	N. P.	Re-injected April 19, 1916 Carrier
N. P.	0.2 x 0.2	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
1.6 x 1.6	0.5 x 0.5, R. I.	N. P.	N. P.	Re-injected April 25, 1916 Carrier
1.6 x 1.6	N. P.	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
1.8 x 1.8, diffuse R. I.	0.2 x 0.2	N. P.	N. P.	Re-injected April 27, 1916 Carrier
0.5 x 0.5, R. I.	0.2 x 0.2	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
0.2 x 0.2, R. I.	0.4 x 0.4	N. P.	N. P.	Re-injected April 25, 1916 Not a carrier
0.2 x 0.2, R. I.	0.1 x 0.1	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
D. R. A.	1.3 x 1.3	1.1 x 1.1	N. P.	
1 x 1, red	1.6 x 1.6	0.2 x 0.2	N. P.	
Red spot	Red spot	Red spot	N. P.	
D. O.	2.1 x 2.1	D. O.	N. P.	
N. P.	1.8 x 1.8	0.2 x 0.2	N. P.	
N. P.	N. P.	N. P.	N. P.	
Sl. red spot	1.6 x 1.6, W. D.	N. P.	N. P.	
Red spot	1.4 x 1.4	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	

KEY TO TABLES—*Continued*

D. R. I. = diffusely indurated and red
D. R. area or D. R. A. = diffuse red blush
S. spot = small spot
N. P. = needle puncture, traumatic reaction
I. R. = indurated red area
D. I. = diffuse induration

TABLE 3.—(*Continued*)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN

Animal	Immunization	Last Injection (1915)	Serologic Tests Jan. 27, 1916		Hours	Typhoidin 3	
			Agglutination	Complement-Fixation		1:100	1:500
342	Vaccine B	Nov. 16, 64 days	1:800	0.05	18	D. O.	1.2 x 1.2
					28	1.9 x 1.9, R. I.	Red spot
					54	Red area	N. P.
						(cm.)	(cm.)
343	Vaccine B	Nov. 16	1:2000	0.05	18	1.8 x 1.8, R. I.	
					28	1.1 x 1.1, R. I.	
					54	N. P.	
344	Vaccine B	Nov. 16	1:1000	0.05	18	1.4 x 1.4	1 x 1
					28	1.2 x 1.8	0.5 x 0.5
					54	Red area 0.2	N. P.
346	Vaccine C	Nov. 30	1:2000	0.005	18	1.5 x 1.5	1.2 x 1.2
					28	1.8 x 1.8	0.8 x 0.8, W. D.
					54	0.4 x 0.4	Small red area 0.3
347	Vaccine C	Nov. 30	1:1000	0.005	18	2.2 x 2.2, W. D.	
					28	1.8 x 1.8	
					54	0.6 x 0.6	
348	Vaccine D	Nov. 30	1:1000	0.005	18	1.8 x 1.8, W. D.	
					28	2 x 2, indurated	
					54	N. P.	
349	Vaccine D	Nov. 30	1:1000	0.005	18	1.5 x 1.5, W. D.	1.5 x 1.5, S. I.
					28	1.5 x 1.5	1.5 x 1.5, S. I.
					54	N. P.	N. P.
350	Vaccine D	Nov. 30	1:800	>0.005	18	2.2 x 2.2,	0.2 x 0.2,
					28	2 x 2	N. P.
					54	0.6 x 0.6	N. P.
449	Control		1:40	0.1	18	0.2 x 0.2	N. P.
					28	N. P.	N. P.
					54	N. P.	N. P.
585	Control		1:10	0.05	18	0.6 x 0.6, R. I.	N. P.
					28	0.1 x 0.1	N. P.
					54	N. P.	N. P.
317	B. typhosus Rawlings, 7 injections	Nov. 8	1:20,000	0.0005	18	1.1 x 1.1, yellow center	1.1 x 1.2, R. I.
					48	1.3 x 1.3, I. R.	Red spot
319	B. typhosus Olsen, 7 injections	Nov. 8	1:10,000	0.0001	18	1.6 x 1.6, W. D.	1.6 x 1.6, I. R.
					48	1.3 x 1.3, I. R.	1.4 x 1.4, I. R.

TABLE 3.—(*Continued*)

Results of Intracutaneous Tests with Typhoidin and Fowl Typhoidin

Typhoidin 3	Fowl-Typhoidin 3		Control Powder 3	Remarks
1:1000	1:100	1:1000		
Red spot	D. O.	N. P.	0.2 x 0.2	
Red spot	1.5 x 1.5	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
(cm.)	(cm.)	(cm.)	(cm.)	
1.2 x 1.2	1.2 x 1.2	N. P.	N. P.	
D. R. A.	1.1 x 1.1, R. I.	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
N. P.	1 x 1	0.1 x 0.1	0.2 x 0.1	
N. P.	0.2 x 0.2	N. P.	N. P.	
Edema	N. P.	N. P.	N. P.	
0.2 x 0.3	1.8 x 1.8	N. P.	N. P.	
0.2 x 0.2	1.6 x 1.6, R. I.	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
1.5 x 1.5	1.5 x 1.5	0.3 x 0.5	N. P.	
0.6 x 0.6	1.5 x 1.5	0.2 x 0.1	N. P.	
N. P.	N. P.	N. P.	N. P.	
0.9 x 0.9	1.5 x 1.5, red	N. P.	N. P.	
1 x 1	1.6 x 1.6, red	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
0.8 x 0.8	1 x 1	0.2 x 0.2	0.2 x 0.2	
0.2 x 0.2	0.5 x 0.5	0.2 x 0.2	0.2 x 0.2	
N. P.	N. P.	N. P.	N. P.	
N. P.	0.2 x 0.3	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
N. P.	0.9 x 1	N. P.	N. P.	
N. P.	0.1 x 0.1	N. P.	N. P.	
N. P	N. P.	N P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	

	Fowl-Typhoidin 3, 1:10			Control Powder 3	
D. R. A.	1.1 x 1.2	1.4 x 1.4, not raised, diffuse	1.8 x 1.8, R. I.	N. P.	Re-injected Jan. 25, 1916 Not a carrier
N. P.	Red spot	N. P.	2.2 x 2.2 R. I.	N. P.	
1.6 x 1.6	1.8 x 1.8	N. P.	2.2 x 2.2	N. P.	Re-injected Jan. 25, 1916 Carrier
1.6 x 1.6, I. R.	1 x 1 I. R.	N. P.	1.5 x 1.5, R. I.	N. P.	

TABLE 3.—(*Continued*)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN

Animal	Immunization	Last Injection (1915)	Serologic Tests Jan. 27, 1916		Hours	Typhoidin 3	
			Agglutination	Complement-Fixation		1:100	1:500
321	B. typhosus Dorset, 7 injections	Nov. 8	1:20,000	0.0005	18	1.5 x 1.5, W. D.	1.5 x 1.5, diffuse I. R.
					48	1.6 x 1.6, I. R.	1 x 1 I. R.
407	Bacillus of fowl typhoid, Strain Smith, 7 injections	Dec. 16	1:200	>0.003	18	N. P.	N. P.
					26	N. P.	N. P.
					48	N. P.	N. P.
411	B. pullorum, 18 injections	Sept. 22	1:100	0.1	18	2.4 x 2.4, I. R.	D. O.
					48	1.6 x 1.6, I. R.	Red area
427	Bacillus of fowl typhoid, D 4, 17 injections	Sept. 22	1:200	<0.01	18	(cm.) 2.2 x 2.2, W. D.	(cm.) 0.8 x 0.8
					26	1.5 x 1.8, W. D.	0.2 x 0.2
					48	1.7 x 1.7	N. P.
491	B. dysenteriae Mt. Desert (living), 11 injections	Nov. 14	1:50	0	18	1.8 x 1.8	1.1 x 1.1, R. I.
					26	1.9 x 1.9, R. I., W. D.	1.2 x 1.2, R. I.
					48	N. P.	N. P.
556	Control		1:20	0.1	18	N. P.	N. P.
					48	0.2 x 0.4	Edema
					72	N. P.	Edema
568	Bacillus of fowl typhoid A. P. (living), 6 injections	Dec. 16	>1:100	>0.005	18	0.5 x 0.5 R. I.	N. P.
					26	1 x 1, R. I.	N. P.
					48	Small nodule with yellow center	Edema
576	Army vaccine, 3 injections	Dec. 15	1:80	0.05	18	1.6 x 1.6, W. D.	N. P.
					48	1.1 x 1.1, W. D.	N. P.
598	Control		1:20	0.05	18	1.2 x 1.2, R. I.	N. P.
					48	0.2 x 0.2	N. P.
					72	N. P.	N. P.

Medical Corps) at 10-day intervals. The number of organisms employed was the same as was generally used for the prophylactic immunization of man. Forty-seven and 62 days, respectively, after the last injection, the serum of these animals gave decided agglutination and complement-fixation.

Three rabbits (342, 343, 344) were immunized on alternate days with sensitized vaccine sediment. One cubic centimeter of the regular preparation of the California state board of health was given intravenously. The sera of these showed, 76 days after the last injection, some complement-fixation with a

TABLE 3.—(*Continued*)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN

Typhoidin 3	Fowl-Typhoidin 3			Control Powder 3	Remarks
1:1000	1:100	1:1000	1:10		
1.5 x 1.5, indurated	1.8 x 1.8, R. I.	N. P.	1.6 x 1.6, W. D.	N. P.	Re-injected Jan. 25, 1916
N. P.	N. P.	N. P.			
N. P.	N. P.	N. P.			
N. P.	N. P.	N. P.			
N. P	0.2 x 0.2	N. P.	1 x 1	N. P.	
N. P.	Red area with edema	N. P.	0.2 x 0.2	N. P.	
N. P.	N. P.	N. P.		0.3 x 0.5	
(cm.)	(cm.)	(cm.)	(cm.)	(cm.)	
1.6 x 1.6	2 x 2, R. I.	1.5 x 1.5		0.3 x 0.3	Died on re-injection
0.9 x 0.6, D. R. A.	2 x 2, R. I.	1.8 x 1.8, R. I.		N. P.	
N. P.	1.9 x 1.9	0.2 x 0.5, R. I.		N. P.	
1.2 x 1.2, R. I.	1 x 1	N. P.		N. P.	
0.8 x 0.8	1 x 1	N. P.		N. P.	
N. P.	N. P.			N. P.	
N. P.	0.6 x 0.6, Red area	N. P.		N. P.	
N. P.	0.3 x 0.3, Red area	N. P.		Edema	
Edema	N. P.		1.5 x 1.5, R. I.	N. P.	
N. P.	0.2 x 0.2, N. P.	N. P.		N. P.	On re-injection did not become a carrier
N. P.		N. P.		N. P.	
Edema	N. P.	Edema		N. P.	
N. P.	0.8 x 0.2, R. I.	N. P.		N. P.	Rabbit anemic
1.1 x 1.1, R. I.	N. P.	N. P.		N. P.	
N. P.	N. P.	N. P.		N. P.	
N. P.	N. P.	N. P.		N. P.	
N. P.	N. P.	N. P.		N. P.	

polyvalent typhoid antigen, and probably on account of repeated bleedings or incomplete sensitization of the vaccine, a fairly high agglutination titer.

Five rabbits (346, 347, 348, 349, 350) were injected with commercial typhoid vaccines. Two animals (346 and 347) were treated with a polyvalent typhoid-paratyphoid vaccine, and 3 rabbits (348, 349, 350) with a monovalent vaccine of the Army type. All the animals were injected at intervals of 10 days and exhibited, 62 days after the last injection, a fair degree of immunity, as judged from the serum reactions.

Five rabbits (407, 411, 427, 491, 568) were immunized over a long period with various dead and living fowl-typhoid organisms, with B. pullorum, and with B. dysenteriae (Mt. Desert). Some animals (411, 427 and 491) received between 10 and 18 injections, and produced highly potent sera for agglutination tests. One hundred, 31, and 47 days, respectively, after the last injection their sera contained moderate amounts of agglutinins and complement-fixing antibodies.

The four rabbits (449, 556, 585, 598), serving as controls, were of about the same weight and age as the majority of the immunized animals. None of these animals gave serum reactions indicative of an existing immunity against B. typhosus and fowl typhoid.

The glycerin extract preparations were both made with veal-Chapoteau glycerin broth in deep layers. Typhoidin 3 was monovalent (Strain Olsen, carrier) and had an antigenic value of 100 e.u. per 0.001 gm. The fowl typhoidin, No. 3, was polyvalent (5 strains: B_3, AP, D, B_2, and Smith); 50 c.c. of the culture of each strain were mixed and then concentrated, as usual. The final powder had an antigenic value of 50 e.u. per 0.001 gm. The results of this experiment are summarized in Table 3.

Most of the rabbits immunized against typhoid reacted with 0.001 gm. of typhoidin, whereas 0.0002 and 0.0001 gm. caused slight reaction or none at all. The reactions were well defined with 0.001 gm., the areola becoming well indurated and remaining so for at least 40 hours. In comparison with the controls, an areola over 1 cm. in diameter was considered as positive. Further evidence that the reaction probably resulted from an antigen-antibody combination, is indicated by the observation that Rabbits 314, 317, 319, 321, 325, 326, 339, 340, 342, 349, which gave marked areolae with 0.001 gm., showed similar intensive reactions in the first 28 hours; this was true, at least, with 0.002 gm. of typhoidin, some of these animals reacting also to 0.0001 gm. of the glycerin extract. With the latter amount, which was absolutely inert for normal rabbits, the degree of induration was different from that obtained with higher concentrations of typhoidin. In most instances only a red blush was present, the method and degree of immunization having apparently very little influence on the intensity of the reaction. An existing typhoid infection was indicated by positive reactions with 0.0002 gm. of typhoidin in Rabbits 319 and 321 in contrast with Rabbit 317, which was only highly immunized. We will discuss this important observation later, more in detail.

It would appear, therefore, that there is no constant relation between the intensity of the skin reactions and the agglutination titer or complement-fixing power of the immune sera of these animals.

The response of the rabbits immunized with organisms allied to the typhoid bacillus is, in many respects, interesting. Three (411, 427, 491) reacted intensively with high, as well as with low, concentrations of the powder. Apparently those animals which had received the largest number of injections (411 and 427) developed areolae which even surpassed in diameter those of the typhoid-immune rabbits. Immunization with B. dysenteriae (Mt. Desert), an organism immunologically different from B. typhosus, also produced cutaneous hypersensitiveness to typhoidin preparations. This observation was later confirmed on many other animals and will be the subject of a separate paper. Normal rabbits showed a small areola with 0.001 gm. of typhoidin; only Rabbit 598 developed a small, slightly indurated red area, which disappeared, however, in the first 36 hours. The typhoidin was 49 days old, but apparently contained toxic irritating substances.

In working quantitatively, this nonspecific factor, which we believe is either due to split products or to a high salt concentration, does not need further consideration because the powder was inert on normal rabbits in quantities of 0.0002 gm. and less. The experiments of Group 1 show, furthermore, that individual differences exist in normal rabbits — as far as the inflammatory reactions are concerned, which may result from some of the elements in the precipitated glycerin extracts of B. typhosus. The nature and meaning of this inflammatory reaction and the factors which govern the same are just as important a problem for investigation as is the ability of the skin of the infected or immune animal to react in a specific manner.

Fowl-typhoidin 3, which was used in dilution of 1 : 100 and 1 : 1000, gave reactions similar in intensity to those with typhoidin. Rabbits 407, 427, and 568, which served to control the value of this preparation, reacted as with typhoidin. An intense reaction was found only in Rabbit 427; of the other two animals, 568 gave a slight reaction, with a very concentrated suspension (0.01 gm.). Rabbit 411, immunized with B. pullorum—an organism nearly identical with the fowl-typhoid bacillus—failed to react, and yet the same animal reacted apparently in a specific manner to typhoidin.

Rabbits 323, 324, 325, 326, and 333, immunized with at least 7 injections of one-tenth of an agar slant of various typhoid strains, gave slight or negative reactions. On the other hand, Rabbit 314, which had been immunized with the 'Rawlings' strain, and which had received, on account of the profuse growth of this organism, considerably more bacterial proteins than the other rabbits, as well as Rabbits 317, 319, and 321, which had been inoculated 9 days previously with one-half slant of living typhoid organisms, gave good reactions. The carriers, 319 and 321, reacted slightly better than the hyperimmune rabbits. The diameter of the areola produced by typhoidin in dilution of 1:500 was about the same as of that produced by fowl typhoidin in dilution of 1:100.

The rabbits treated with various vaccine preparations responded well to the intradermal application of fowl typhoidin. The reaction was, in most of the animals, surprisingly similar in intensity to that to the same concentration of typhoidin. However, the positive reaction is apparently not an indication of a high degree of immunity, because on subsequent injections with twice the lethal dose of living fowl-typhoid organisms, several of the animals (340, 346, 349) which gave very marked and well-defined indurated areolae succumbed to the infection. This important observation has been made the subject of subsequent experiments, and will be discussed later.

It has been demonstrated in observations in Group 1 that the dose of 0.001 gm. of typhoidin powder, or 0.1 of a suspension or solution of this preparation in salt solution, produces skin reactions in immune rabbits which are distinct and can be considered as specific in nature.

Animals which had been immunized recently, and those which had been injected with large doses of bacterial protein, gave more intensive reactions than those which had been treated a long time previously and with smaller doses of bacterial preparations. These observations support our contention that such reactions are the result of cutaneous hypersensitiveness due to bacterial protein. That this sensitization is not strictly specific is already indicated by the group or co-reactions which were noted with fowl typhoidin.

We failed to record noteworthy nonspecific reactions in the control animals and concluded, therefore, that fowl typhoidin or typhoidin does not contain sufficient toxic elements to act as irritants on the skin of rabbits. An experiment was therefore planned in which the most favorable conditions might exist for the production of typhoid and fowl-typhoid toxins. Glycerin broth cultures with high peptone content and free exposure to oxygen were tested on a series of rabbits.

EXPERIMENT-GROUP 2

Eleven rabbits were chosen for this experiment. Three (619, 642, 644) had been injected once with a recently isolated culture of B. typhosus. One rabbit (644) had a fair degree of immunity, the other two proved at autopsy to be carriers (liver and gall-bladder). The remaining 8 rabbits (638, 639, 646, 647, 648, 662, 663, 698) had served as controls for some other experiments and had been injected with various typhoidin preparations from 4 to 6 weeks previous to their use in Group 2. This treatment apparently had produced conditions in the skin and blood serum of some of the rabbits which were not anticipated and which therefore made the interpretation of the results very difficult.

The preparation of the typhoidin used in this experiment was as follows: (1) B. typhosus Olsen* was inoculated into glycerin rabbit broth containing 2% Witte's peptone, and grown in shallow layers in several Blake bottles for 5 days. The contents of the various bottles were mixed, extracted at 60 C., concentrated, and precipitated as usual. A fowl typhoidin (Strain 605) was prepared in the same manner. These glycerin extracts were 3 days old when used.

(2) Typhoid and fowl-typhoid bacilli were similarly inoculated into Martin's peptone broth and grown in Blake bottles. Before concentration, sufficient pure glycerin was added to the mixture to make a 5% solution. A comparatively small amount of precipitate was obtained from these cultures, yet the antigenic value was about the same as for the other preparation described.†

As controls, we used sterile broth preparations treated in identical manner.

The results of this interesting test are shown in Tables 4a and 4b.

The preparations Gɪ and Gɪɪ produced, as will be noted, in most animals (except 662 and 663) very severe skin reactions, the degree of induration and diameter of the areola depending entirely on the amount of powder-suspension inoculated. Previous tests, or the amount or type of typhoidin applied intracutaneously, had no influence on the present degree of the reactions. The typhoidin was somewhat more intense than the fowl typhoidin. Unfortunately, only one rabbit (684) acted as a control and, judging from this single observation, the powder Gɪ was slightly irritant to an unsensitized rabbit.

The infected rabbits (619 and 642) gave positive skin tests which persisted for 48 hours. In comparison with the large edematous areola of the sensitized rabbits and the immune rabbit (644), the reactions were small. In the light of some other experiments, to be discussed later, this result—for which we offer 2 possible explanations—was unexpected. Either the typhoidin preparation Gɪ was detoxicated in some of the rabbits possessing a certain degree of immunity (Rabbits 644 and 663) or, what is perhaps more plausible, the anemic, emaciated, and infected animals (619, 642) for reasons thus far not investigated, responded poorly to cutaneous tests. We may recall here that similar observations have been made in tuberculinization of man and animal. Calmette, Breton, and Petit[31] found a disappearance of the ophthalmic reaction with the beginning of cachexia in tuberculous rabbits.

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* Gɪ=typhoidin in 2% peptone broth = 20 e. u. per 0.001 gm. Gɪɪ = fowl typhoidin in 2% peptone broth = 10 e. u. per 0.001 gm.

† Fɪ = Martin's-broth typhoidin = 20 e. u. per 0.001 gm. Fɪɪ = Martin's-broth fowl typhoidin = 33 e. u. per 0.001 gm.

[31] Compt. rend. Soc. de biol., 1907, 63, p. 296.

TABLE 4a

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN
DATE OF TEST: MAY 19, 1916

Animal	Infection and Result	Previous Test	Serologic Test, May 17, 1916		Hours
			Agglutination	Complement-Fixation	
619	B. typhosus H-125 carrier May 8, 1916		1:4000 —1:8000	0.003	24 48 72
638	Control	April 10, 1916 Typhoidin P.C. Paratyphoidin A Paratyphoidin B B. coli	1:100	0.05	24 48 72
639	Control	April 10, 1916 Typhoidin P.C. Paratyphoidin A Paratyphoidin B B. coli	1:40	0.1	24 48 72
644	B. typhosus H-125 April 13, 1916		1:1000 —1:2000	0.001	24 48 72
685	Control		1:20	0.1	24 48 72
698	Control	April 10, 1916 Typhoidin P.C. Paratyphoidin B Paratyphoidin A B. coli	1:40	0.05	24 48 72

The control powders, rich in peptones, were not absolutely inert; small indurated areolae developed with broth-control suspensions, becoming smaller, however, after 24 hours and even disappearing entirely in rabbits of poor reaction. Some rabbits seemed to respond more intensely than others, individual factors appearing to play a certain rôle. The possible criticism that the numerous skin tests on one and the same animal may influence one another, is not substantiated by our observations.

Preparations Fi and Fii, probably on account of the smaller peptone content, were less active in previously sensitized rabbits, yet not entirely inert on normal rabbits. We have already referred to the fact that, unfortunately, only one control rabbit was used in each lot of Group 2. It is not unlikely that Rabbit 685 was one of those susceptible animals which gave a very marked skin areola with edema following the injection of Typhoidin Fi, a response which we have already described in Group-experiment 1. The presence of a marked

TABLE 4a—(*Continued*)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN
DATE OF TEST: MAY 19, 1916

Typhoidin F		Control Powder	Fowl-Typhoidin G II		Control Powder
1:100	1:50		1:100	1:50	
(cm.)	(cm.)	(cm.)	(cm.)	(cm.)	(cm.)
1.2 x 1.2	1.4 x 1.4	N. P.	0.9 x 0.9	1.5 x 1.5	N. P.
1.3 x 1.3	1.8 x 1.8	N. P.	1.1 x 1.1	2.1 x 2.1	N. P.
N. P.	N. P.	N. P.	N. P.	N. P.	N. P.
2.7 x 2.7	3.8 x 3.8, raised 1.2	1 x 1	1.7 x 1.7	2.1 x 2.5	0.9 x 0.9
2.8 x 2.8	2.1 x 2.1, R. I.	0.5 x 0.5	0.9 x 0.9	2.4 x 2.5	0.6 x 0.6
N. P.	0.5 x 0.5, nodule	N. P.	N. P.	0.6 x 0.6	N. P.
2.4 x 2.4	2.9 x 2.9	1 x 1	1.4 x 1.4	1.5 x 1.5	0.9 x 0.9
1.8 x 1.8	2 x 2	0.5 x 0.5	0.5 x 0.5	0.8 x 0.8	0.8 x 0.8
0.7 x 0.7	1.3 x 1.3	0.5 x 0.5	N. P.	0.6 x 0.6	N. P.
2.5 x 2.5	2.2 x 2.8	0.8 x 0.8	1.5 x 1.5	2.3 x 2.3	0.8 x 0.8
2 x 2	2.6 x 1.9	N. P.	0.7 x 0.7	0.8 x 0.8	0.6 x 0.6
N. P.	0.8 x 0.8, nodule	N. P.	N. P.	0.8 x 0.8, nodule	N. P.
Diffuse edema	Diffuse edema	N. P.	1 x 1	1.1 x 1.1	0.8 x 0.8
1.6 x 3.1, edema	1.6 x 3.1	N. P.	0.6 x 0.6	1.4 x 1.4	0.5 x 0.5
N. P.	N. P.	N. P.	N. P.	N. P.	N. P.
3 x 3	3.4 x 3.4	0.9 x 0.9	1.6 x 1.6	2 x 2	1 x 1
2.5 x 2.5	2 x 2.5	0.6 x 0.6	1.6 x 1.6	2.2 x 2.2	0.7 x 0.7
1.4 x 1.4	1 x 2.5	N. P.	0.5 x 0.5	1.3 x 1.3	0.6 x 0.6

edema with slight hyperemia, induration, and increased heat is in striking contrast, however, with the typical cutaneous reactions in sensitized rabbits. Most of the rabbits reacted simultaneously to fowl typhoidin, and the criticism that some of the reactions were due to faulty intracutaneous technic and dosage of the suspensions is not well supported by the quantitative tests. The accuracy of our technic is shown by the fact that the areolae and the degrees of induration were dependent on the concentrations of the various powders. Small reactions with typhoidin, or their entire absence, were paralleled by a similar response with fowl typhoidin.

Analyzing Tables 4a and 4b more carefully, we note that previous injections of typhoidin produced in a certain percentage of animals a condition of the skin which resulted in the development of severe reactions subsequent to a new intracutaneous application of typhoidins rich in peptones and toxic split products. In our opinion, 2 factors are responsible for these conditions: in the first place it is possible that

TABLE 4b

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN
DATE OF TEST: MAY 19, 1916

Animal	Infection and Result	Previous Test	Serologic Test, May 17, 1916		Hours
			Agglutination	Complement-Fixation	
642	Control	April 13, 1916 B. typhosus H-125 I. V., carrier	1:2000	>0.003	24 48 72
646	Control	April 17, 1916 Typhoidin 15 Fowl-typhoidin 4	1:40	0.05	24 48 72
647	Control	April 17, 1916 Typhoidin 15 Fowl-typhoidin 4	1:40	0.1	24 48 72
648	Control	April 17, 1916 Typhoidin 15 Fowl-typhoidin 4	1:100 —1:200	0.05	24 48 72
662	Control	April 25, 1916 Typhoidin E Paratyphoidin A Paratyphoidin B	1:40	0.05	24 48 72
663	Control	April 25, 1916 Typhoidin E Paratyphoidin A Paratyphoidin B	1:200 —1:400	>0.05	24 48 72
684	Control		1:20	0.1	24 48 72

a fair number of rabbits are capable of developing cutaneous hypersensitiveness to typhoidin and similar preparations—a response which is probably a phase of a general sensitization; or, secondly, on account of some individual factors or on account of the unsuitability of the preparations used, not every animal may react to repeated skin tests. Rabbits 662 and 663 illustrate the point that the typhoidin preparations used for sensitization may have some influence because, in contrast with Rabbits 646, 647, and 648, the reactions in these two rabbits were small. But it should be kept in mind that individual differences also play a part in the explanation of the intensities of the reactions noted in Rabbits 662 and 663.

The second factor, the hypersensibility of the rabbit skin to peptone and toxic split products also merits consideration. It is difficult to decide how regularly the condition occurs in animals until a large series of tests has been carried out. From experience with tuberculin

TABLE 4b.—(*Continued*)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN
DATE OF TEST: MAY 19, 1916

Typhoidin G I		Control Powder	Fowl-Typhoidin F II		Control Powder
1:100	1:50		1:100	1:50	
(cm.)	(cm.)	(cm.)	(cm.)	(cm.)	(cm.)
1.8 x 1.8	2 x 2	N. P.	1.5 x 1.5	1.8 x 1.8	0.7 x 0.7
0.9 x 0.9	1.4 x 1.4	0.4 x 0.4	0.9 x 0.9	1.6 x 1.6	0.6 x 0.6
Red spot	0.7 x 0.7	N. P.	N. P.	N. P.	N. P.
3.2 x 3.2	3.7 x 3.7, raised 0.5	0.9 x 0.9	1.2 x 1.2	1.8 x 1.8	0.8 x 0.8
1.8 x 1.8	2.3 x 2.9	N. P.	1.1 x 1.1	1.6 x 1.6	0.5 x 0.5
0.3 x 0.3	0.8 x 0.8, indurated	Red spot	Red spot	Red spot	Red spot
2.9 x 2.9, edema; R. I.	3.6 x 3.6	1 x 1	17. x 1.7	1.4 x 1.4	0.9 x 0.9
1.5 x 1.5	1.6 x 1.6, indurated	0.8 x 0.8	1.8 x 1.8	1.9 x 1.9, I. R.	0.9 x 0.9
0.3 x 0.3	0.5 x 0.5	Red spot	Red spot	0.3 x 0.3	Red spot
2.4 x 2.4	3.5 x 3.5	0.8 x 0.8	1.8 x 1.8	2 x 2	0.6 x 0.6
1.4 x 1.4	1.5 x 2.7	0.6 x 0.6	1.2 x 1.2	2 x 2	0.8 x 0.8
0.5 x 0.5	1.1 x 1.1	N. P.	0.4 x 0.4	0.4 x 0.4	Red spot
0.5 x 0.5	3.6 x 3.6, edema	0.6 x 0.6	1.3 x 1.3	N. P.	0.6 x 0.6
0.3 x 0.3	0.8 x 0.8	0.8 x 0.8	N. P.	N. P.	N. P.
0.2 x 0.2	0.5 x 0.5, R. I.	0.3 x 0.3	N. P.	N. P.	N. P.
0.8 x 0.8	N. P.	N. P.	0.6 x 0.6	N. P.	N. P.
0.4 x 0.4	0.4 x 0.4	N. P.	1 x 1	1.6 x 1.6	0.3 x 0.3
0.5 x 0.5	Red spot	N. P.	0.3 x 0.3	0.2 x 0.2	N. P.
0:5 x 0.5	0.6 x 0.6	N. P.	N. P.	N. P.	N. P.
0.2 x 0.2	0.4 x 0.4	N. P.	N. P.	N. P.	N. P.
N. P.	N. P.	N. P.	N. P.	N. P.	N. P.

we know that the rabbit is about three million times less sensitive to this preparation than is man. Preparations of bacterial extracts which give, in low dilutions, large nonspecific reactions in man are, as a rule, absolutely inert in the rabbit. Our observations in Experiment-group 2 would indicate that a hypersusceptibility of rabbits to peptone may occur, but in a limited percentage of animals only. When, however, preparations with the regular low peptone content are used, we have observed misleading reactions in rare instances.

From the experiments of Group 2 we may conclude that 5-day-old glycerin extracts of the typhoid and the fowl-typhoid bacillus containing 2% Witte's peptone or made from pig stomach and kept in shallow layers, contain a fair amount of antigenic substances. The intense reactions produced by them on immunized and previously 'typhoidinized' rabbits are, however, out of proportion to the antigenic value of the preparation. These preparations also cause nonspecific edemas and reactions in normal rabbits and are therefore considered unsuitable for cutaneous tests.

It may be assumed that these preparations are irritant and nonspecific primarily on account of the high endotoxin content which we know from previous experiments may develop in this type of medium and under the conditions of free exposure to oxygen, and as a result of the high concentration of alcohol-precipitable proteids. These substances—peptones, albumoses, and other protein split products—in all probability favor also the alcohol precipitation of the endotoxin by adsorption. As a rule, typhoid toxins or similar toxic protein split products are, according to von Stenitzer,[32] not quantitatively precipitable by alcohol. In addition to these conditions, all these undesirable precipitable substances considerably dilute the antigenic substances demonstrable by fixation tests and probably are in part responsible for the low antigenic value of the extract (1 mg. of the typhoidin G and F).

The observations made in Group 2 suggested the use of glycerin extracts of young cultures in which the toxin-production is just commencing and which is, therefore, of low concentration. Furthermore, it was considered advisable to use different strains of B. typhosus for these cultures. Old stock cultures ordinarily grow very vigorously in glycerin veal broth, and the precipitate obtained is abundant in comparison with the poor growth of recently isolated strains. Also the toxogenic effect of various strains in one and the same medium is, in our opinion, not uniform. A low toxic strain with optimal growth would constitute the best organism for a suitable typhoidin. Pulay[2] suggested in his publication, the use of a 48-hour-old culture of a recently isolated strain. In his experience a 'typhin' prepared in this manner gave better and more distinct reactions than one made with an old strain.

In two series the value of these suggestions was tested. Extracts of other organisms were included to determine the sensitiveness of the immunized rabbits in general. In one series B. coli and B. dysenteriae, and in one, staphylococci and fowl-typhoid extracts, were used.

EXPERIMENT-GROUP 3

Two lots, of 4 and 5 rabbits, respectively, together with 2 controls, were tested with various preparations on Feb. 23, 1916.

Lot I consisted of 4 rabbits (332, 337, 358, 360) which were immunized against

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[32] Handbuch d. Technik und Methodik d. Immunitätsforschung, 1908, 1, p. 200.

4 different strains of typhoid bacilli. Fifty and 109 days, respectively, following the last injections, the animals were in good health and, as could be judged by the serum reactions, still showed a fair degree of immunity.

The glycerin extracts used had the following antigenic value: Typhoidin 4 (Kuhn) 48 hours' growth (strain isolated in November, 1915), 10 e.u. per milligram.

Fowl-Typhoidin 4, (A. P. Smith) 5 days' growth, 50 e.u. per milligram. The 'staphylococcin' was prepared from a 48-hour-old culture of a recently isolated human strain of staphylococcus; its antigenic value was not determined. All three extracts used were prepared with a veal Witte's peptone glycerin (5%) broth. The results of the skin tests are shown in Table 5.

A glycerin extract prepared from a 48-hour-old culture of a recently isolated strain of B. typhosus with fair antigenic properties, caused, on intradermal injection of 1 mg., reactions which differed very little—if at all—from those produced in the controls. On the other hand, a fowl typhoidin prepared from a 5-day-old culture at the same time and with the same medium, caused the formation of areolae similar in size and intensity to those already described in the experiments of Group 1; the intensity of the reactions varied somewhat in different animals. The staphylococcus extract, which was absolutely inert as tested on several other control animals not mentioned in the protocol, caused in one rabbit (358) a well-defined, typical reaction which persisted for 48 hours. A small areola was also noticed in Rabbit 332, but as will be seen from Table 5, the reaction to fowl typhoidin in this animal remained for at least 48 hours, those to the staphylococcus extract having by that time nearly disappeared. An explanation of this nonspecificity, which is observed in a certain percentage of rabbits, will be attempted later when similar or identical observations with other extracts have been cited.

In Lot 2, 5 rabbits (335, 336, 355, 357, 361) had been immunized with varying amounts of typhoid antigens of different strains. Corresponding with the lapse of time (58, 102, and 107 days) after the last injection, the immunity was still well marked. One animal (357) suffered from a coccidiosis relapse and was emaciated and anemic on the day of the test; all the other animals were in good condition. Rabbits 335 and 336, which were older animals than 355 to 361, had been immunized with very small doses of very toxic, recently isolated strains.

Only extracts of 48-hour-old cultures in a medium of the same composition as that used on Lot 1, were used. The typhoidin Rawlings (Army strain) had an antigenic value of 20 e.u. per milligram. The dysentery strain, Shiga Do., was an old laboratory strain received from the Institut Pasteur, whereas the colon bacillus had been recently isolated from a case of cystitis. The results of the test are shown in Table 6.

TABLE 5

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN, STAPHYLOCOCCIN AND FOWL TYPHOIDIN
DATE OF TEST: FEB. 23, 1916

Animal	Immunization	Last Injection, 1915	Serologic Tests		Hours	Typhoidin 'Kuhn' (48)	
			Agglutination	Complement-Fixation		1:100	1:500
						(cm.)	(cm.)
332	B. typhosus 46, 7 injections	Nov. 8	1:800	0.003	16	0.5 x 0.5	0.2 x 0.2
					24	N. P.	N. P.
					48	N. P.	N. P.
337	B. typhosus 48, 7 injections	Nov. 8		0.005	16	0.2 x 0.2	N. P.
					24	0.2 x 0.2	N. P.
					48	N. P.	N. P.
358	B. typhosus 79, 7 injections	Dec. 26	1:800	0.01	16	0.8 x 0.8	0.3 x 0.3
					24	0.6 x 0.6, R. I.	N. P.
					48	N. P.	N. P.
360	B. typhosus 90, 7 injections	Dec. 26	1:800	0.005	16	0.4 x 0.4	0.2 x 0.2
					24	0.5 x 0.5, S. I.	N. P.
					48	N. P.	N. P.
607	Control		1:40	0.1	16	0.3 x 0.3	N. P.
					24	N. P.	N. P.
					48	N. P.	N. P.

In Lot 2 the precipitate of a 48-hour-old typhoid culture of the Rawlings strain produced slight or no reactions at all in properly immunized animals. Only Rabbit 335 gave a reaction which could be considered positive. The striking absence of the least reaction in Rabbits 355, 357, and 361 is difficult to explain. The absence of reactions with other bacterial extracts suggests that either Rabbits, 355, 357, and 361 possessed no cutaneous hypersensitiveness, or that the test preparations were insufficiently active in the 1-mg. amounts injected. The first explanation is not very well supported by subsequent observations in which the same rabbits gave good and distinct reactions; it is apparent, however, that their sensitiveness must have been low, either on account of disease or because of the few injections, inasmuch as Rabbits 335 and 336, which reacted with typhoidin, gave very marked reactions also with the colon-bacillus and dysentery extracts. In comparison, the typhoidin areolae were small, but

TABLE 5.—(*Continued*)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN, STAPHYLOCOCCIN AND FOWL TYPHOIDIN
DATE OF TEST: FEB. 23, 1916

Staphylococcin (48) 1:100	Fowl-Typhoidin 4 (5)		Control-Powder 4 1:10	Remarks
	1:100	1:500		
(cm.)	(cm.)	(cm.)	(cm.)	
0.9 x 0.9, R. I. 0.3 x 0.3, R. I. N. P.	1.3 x 1.3, R. I. 1.2 x 1.2, W. D. 1.1 x 1.1, R. I.	0.2 x 0.2 N. P. N. P.	N. P. N. P. N. P.	Re-injected March 2, 1916 Not a carrier
0.3 x 0.3 0.3 x 0.3, diffusely red N. P.	Diffuse red area 2 x 2 1.7 x 1.7, R. I.	1.9 x 1.9, R. I. 1.9 x 1.9, R. I. N. P.	0.3 x 0.3 0.2 x 0.2 N. P.	Re-injected March 2, 1916 Not a carrier
2.1 x 2.1, R. I. 1.9 x 1.9, R. I., W. D. N. P.	1.1 x 1.1 1.1 x 1.1 N. P.	0.2 x 0.2 N. P. N. P.	N. P. N. P. N. P.	Re-injected March 3, 1916 Carrier
0.3 x 0.3 N. P N. P.	1.7 x 1.7, I. R. 2.2 x 2.2, W. D. 1.6 x 1.6, R. I.	Diffuse red area 0.9 x 0.9, I. R. N. P.	N. P. N. P. N. P.	Re-injected March 2, 1916 Carrier
N. P. N. P. N. P.	0.5 x 0.5 0.2 x 0.2 N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	

decidedly better than those produced by the preparation 'Kuhn' used on Lot 1. The absence of skin reactions in these three rabbits is probably the result of several factors combined. It is furthermore possible that the famous Rawling strain, which recently through the studies of Hooker[33] and Garbat[34] has been found to exhibit antigenic properties different from those of recently isolated typhoid strains, may also act abnormally in cutaneous tests. At present our data do not warrant such conclusions.

The positive reactions with extracts of B. coli and B. dysenteriae Do. in Rabbits 335 and 336 are interesting from many viewpoints. Both preparations were practically inert on normal animals. That these reactions were not the result of normal broth constituents is shown by the slight reactions (0.01 gm.) of broth precipitates produced

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[33] Proc. Soc. Exper. Biol. and Med., 1916, 13, p. 139.

[34] Jour. Immunol., 1916, 1, p. 391.

TABLE 6

RESULTS OF INTRACUTANEOUS TESTS IN GROUP 3, LOT 2
DATE OF TEST: FEB. 23, 1916

Animal	Immunization	Last Injection, 1915	Serologic Tests, Feb. 23, 1915		Hours	Typhoidin 'Rawlings' (48)	
			Agglutination	Complement-Fixation		1:100	1:500
						(cm.)	(cm.)
335	B. typhosus 49, 7 injections	Nov. 8	1:600	0.02	16	0.8 x 0.8	0.4 x 0.4
					24	1.2 x 1.2, R. I.	0.9 x 0.9, S. I.
					48	0.5 x 0.5	0.3 x 0.3
336	B. typhosus 50, 7 injections	Nov. 8	1:2000—4000	1.005	16	0.3 x 0.3	0.3 x 0.3
					24	0.5 x 0.5	0.2 x 0.2
					48	N. P.	N. P.
355	B. typhosus 73, 3 injections	Nov. 13	1:1000	0.01	16	N. P.	N. P.
					24	N. P.	N. P.
					48	N. P.	N. P.
357	B. typhosus 75, 6 injections	Dec. 27	1:2000	0.005	16	N. P.	N. P.
					24	N. P.	N. P.
					48	N. P.	N. P.
361	B. typhosus 94, 6 injections	Dec. 27	1:2000—4000	0.005	16	N. P.	N. P.
					24	N. P.	N. P.
					48	N. P.	N. P.
608	Control		1:10	0.05	16	N. P.	N. P.
					24	0.2 x 0.5	N. P.
					48	N. P.	N. P.

on the skin of Rabbits 335 and 336. It is possible that these rabbits were very susceptible to toxic split products produced by B. coli and B. dysenteriae in the culture medium. Against this explanation is the absence of reactions in the control, Rabbit 608. In the light of experiments to be discussed in the next paper, these reactions may be explained as the result of nonspecific sensitization.

These group experiments do not very well support the contention of Pulay, that a 48-hour culture is just as suitable as a 5-day growth, and a recently isolated strain just as good as an old one. Furthermore, it is evident that the amount of extract units(e. u.) demonstrated by the complement-fixation test does not absolutely indicate the value of the preparation as an antigen.

The growth of the typhoid and staphylococcus strains in 48 hours was rather poor, and we suspect that the contents of the precipitate in antigenic bacterial proteins was correspondingly small. That the

TABLE 6.—(*Continued*)

RESULTS OF INTRACUTANEOUS TESTS IN GROUP 3, LOT 2

DATE OF TEST: FEB. 23, 1916

B. Dysenteriae Shiga (48) 1:100	B. Coli 4 (48)		Control-Powder 4 1:10	Remarks
	1:100	1:500		
(cm.)	(cm.)	(cm.)	(cm.)	
2.2 x 2.2, R. I.	2.2 x 1.5, R. I.	0.9 x 0.9	0.2 x 0.2	Carrier
2.1 x 2.1, R. I.	1.8 x 1.8, I. R.	1.1 x 1.1	0.2 x 0.2	
1.8 x 1.8, S. I.	1.9 x 1.9, R. I.	N. P.	N. P.	
1.9 x 1.9, R. I.	2 x 2, D. I.	0.9 x 0.9	0.2 x 0.2	Re-injected March 2
2.5 x 2.5, R. I.	1.5 x 1.5, S. I.	1.5 x 1.5	0.2 x 0.2	Carrier
1.5 x 1.5, R. I.	0.6 x 0.6, R. I.	0.2 x 0.2	N. P.	
N. P.	N. P.	N. P.	N. P.	Re-injected March 3
N. P.	N. P.	N. P.	N. P.	Died intercurrent disease
N. P.	N. P.	N. P.	N. P.	Not a carrier
N. P.	N. P.	N. P.	N. P.	Died on February 25
N. P.	N. P.	N. P.	N. P.	Anemic and sick when tested
N. P.	N. P.	N. P.	N. P.	Emaciated
N. P.	N. P.	N. P.	N. P.	Re-injected March 2
N. P.	N. P.	N. P.	N. P.	Became carrier
N. P.	N. P.	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
0.8 x 0.8	N. P.	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	

growth of the organism was slight is quantitatively evidenced by the weights of the precipitates.*

It is also quite possible that the insufficient growth of the microorganisms did not produce those substances in the broth cultures which are essential for a distinct skin reaction. One point is quite certain from observations on Lots 1 and 2 of this group, and that is that a good fowl typhoidin will produce skin reactions in typhoid-immune rabbits, and that a few of these animals will also give reactions with extracts of B. coli, staphylococci, and B. dysenteriae.

SUMMARY

In this communication on cutaneous tests with glycerin extracts of broth cultures, we have endeavored to explain our methods of preparation of the antigenic powders. These studies have been made, with a few exceptions, entirely with glycerin extracts of broth cultures. In the course of our observations it became clear that glycerin is not absolutely necessary for the preparation of good antigens, but we felt nevertheless that for comparative tests experimental data should first be collected with a preparation already known and analogous to the tuberculin and mallein, with which we have had considerable experience.

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* Weight of dried precipitate of 10 c.c. of concentrated glycerin extract: B. typhosus (Kuhn), 48 hours' growth = 0.3990 gm. B. coli, 48 hours' growth = 0.4813 gm. B. dysenteriae Shiga, 48 hours' growth = 0.4442 gm. Staphylococcus, 48 hours' growth = 0.3890 gm. B. typhosus, No. 4 (Olsen), 5 days' growth = 0.6931 gm.

In this study we have attempted also to obtain some information regarding the composition and reaction of the medium, the growth of the bacteria and their toxic split products, the age of the culture, the type of strain, and other factors with reference to the influence they may have on the suitability of the resulting product for these tests.

It has been found, for instance, that a high peptone content together with rich exposure of the broth to oxygen, will produce typhoidin powders which are very irritant and active for nonimmune or sensitized rabbits. Young 48-hour-old cultures of recently isolated strains, on the other hand, produce powders which are inactive in dilutions ordinarily suitable for old (5- to 15-day) cultures. Vigorously growing old laboratory strains of the typhoid bacillus and other bacteria give, at times, active antigenic powders.

With the desire to reduce the production of toxic split products and to increase the antigenic substances, we used completely filled flasks containing potato broth. When the bacteria were grown in this manner, the protein-sparing action of the carbohydrates and the long incubation of the cultures resulted in extracts which were practically atoxic as tested by intravenous inoculation of sterile filtrates (in several tests only amounts of from 8 to 10 c.c. produced typical death). The studies of Theobald Smith and Ten Broeck[35] on the fowl-typhoid toxin, those of Kraus and Stenitzer[36] on the typhoid toxin, and our findings with the B.-enteritidis and B.-paratyphoid-A toxins, suggested the use of cultures over 10 days old, because, as a rule, the maximal amount of toxin is produced between the 8th and the 10th day.

Experience has shown that fractional extractions at a temperature increasing from 40 to 60 degrees, resulted, just as in the case of tuberculin, in preparations which were more active than those concentrated rapidly at high temperatures. Filtration and rapid treatment with alcohol, favor the production of salt-solution-soluble powders. Unfiltered extracts, or those washed repeatedly with alcohol, are frequently insoluble. In form of suspensions such preparations act nonspecifically as irritants.

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[35] Jour. Med. Research, 1914-1915, 31, p. 523.

[36] Ztschr. f. Immunitätsf., 1909, 3, p. 646.

We recommend the titration of the glycerin extracts by means of the complement-fixation test, primarily to determine the antigenic value and, secondarily, to follow the deterioration of a preparation. We have not as yet made full use of this method, but we record a few suggestive observations. The value of typhoidin or broth-culture extract should also be tested as to its sensitizing value by inoculations into rabbits in a dosage of 0.1 to 0.01 gm. It is of interest to bear in mind, however, that powders perfect in every respect may prove on animal inoculation to possess low antigenic properties.

In quantities of 0.001 to 0.0002 gm. a perfect preparation was found to be inert on the skin of normal untreated rabbits. In immunized, infected, or otherwise sensitized animals, typical inflammatory areolae—characterized by hyperemia, induration, and increased warmth—developed at the site of injection. Central papules with yellowish centers developed only with very concentrated and insoluble powders, and are in our experience the result of pyogenic action of the concentrated bacterial proteins. It is self-explanatory that such preparations are unsuitable for careful skin tests. In our experiments we successfully used various dilutions of one antigenic powder. These quantitative tests not only control the technic of injection, but they also yield, in many instances, information concerning the gradual diminution in the intensities of the reactions, thus making decisions possible in regard to specificity.

The use of control powders in fairly high concentration — which in reality demonstrate only the individual susceptibility of the rabbit skin to peptone and broth elements—was amplified by control powders which were made with various other bacterial cultures, as B. coli, B. dysenteriae, etc. The reactions which resulted from the use of such powders indicated more precisely the status and nature of the cutaneous hypersensitiveness of a rabbit to bacterial protein. Subsequent papers will deal more extensively with these subjects.

CONCLUSIONS

Glycerin broth-culture extracts of the typhoid bacillus precipitated with alcohol can be used for cutaneous tests on rabbits, provided the culture is poor in peptones and the organism is grown for a long time with a limited exposure to oxygen.

The proper amount of bacterial extract powder to produce distinct reactions in rabbits varies between 0.0001 and 0.0002 gm. The reaction apparently depends on other antigenic substances than those which can be titrated by means of the complement-fixation test.

Powders prepared of the sterile broth serve as controls only for the hypersensitiveness of the rabbit skin to broth precipitates and extractives. Broth cultures of other bacteria are recommended as additional controls.